*Immunology* 1994 **83** 52–57

# ICAM-1/LFA-1 interactions in T-lymphocyte activation and adhesion to cells of the blood–retina barrier in the rat

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## SUMMARY

To identify the signals involved in the adhesion and subsequent migration of lymphocytes across the endothelium (REC) and pigment epithelium (RPE) of the blood–retina barrier we have studied the effects of monoclonal antibodies (mAb) to rat adhesion/accessory molecules on the binding of normal and concanavalin A (Con A)-activated rat spleen lymphocytes to cultured unstimulated and interferon-$\gamma$ (IFN-$\gamma$)-stimulated RPE and REC. Forty to 48% of unactivated T cells were found to bind to normal REC or RPE by leucocyte function-associated antigen-1/intercellular adhesion molecule-1 (LFA-1/ICAM-1)-independent mechanisms, despite constitutive expression of ICAM-1 by the RPE cells and LFA-1 by the T cells. Con A-activated lymphocytes showed an enhanced adhesion to both RPE and REC. However, IFN-$\gamma$-stimulated RPE and REC did not demonstrate a significant increase in adhesiveness for normal lymphocytes highlighting the importance of lymphocyte integrin activation from low-affinity to high-affinity state. Activated lymphocyte adhesion to unstimulated RPE and REC was significantly blocked by LFA-1 mAb (35%, $P < 0{\cdot}0001$) and ICAM-1 mAb (20%, $P < 0{\cdot}001$). Inhibition of adhesion by antibody to CD2 was not significant. Both ICAM-1 and LFA-1 mAb also significantly ($P < 0{\cdot}05$) blocked antigen presentation following retinal extract stimulation of lymphocytes from immunized rats in proliferation assay. These data suggest that the ICAM-1/LFA-1 system is important in lymphocyte trafficking into the eye only after lymphocyte activation.

## INTRODUCTION

Soluble retinal antigens, present in the rod outer segments (ROS) of the retina are potent autoantigens which can induce a $CD4^+$ T-lymphocyte-mediated posterior uveitis in experimental animals (experimental autoimmune uveitis, EAU).[1] EAU in the rat is characterized by destruction of the photoreceptor cells of the retina, where the eliciting antigen(s) is located and lesions such as photoreceptor damage, retinal detachment, vitritis, retinitis, choroiditis and vasculitis, resembling human disease are commonly observed.[1] In posterior uveitis, autoreactive T cells must be activated before they traverse the blood–retina barrier, but how the T cells become activated and the mechanisms by which they migrate into the eye to reach their target are largely unknown. The blood–retina barrier which is analogous to the blood–brain barrier consists of both capillary endothelial cells and retinal pigment epithelial cells. Both cell types have been suggested as potential activators of circulating T cells.[2,3]

In the immune system the process of adhesion is crucial for both lymphocyte activation and for migration to various anatomical sites,[4] multiple intercellular signalling events being required in addition to major histocompatibility complex (MHC)/peptide recognition for antigen presentation and co-stimulation of lymphocyte proliferation.[4] In particular, intercellular adhesion molecule-1 (ICAM-1) and its ligand leucocyte function-associated antigen-1 (LFA-1) have been shown to have an important role, not only in leucocyte adherence but also in transmission of activation signals.[5] Endothelial cells also constitutively express ICAM-2, an alternative ligand for LFA-1,[6] and LFA-3, the ligand for CD2, a co-stimulatory molecule which is present on T cells.[7] LFA-1, the counter receptor for ICAM-1 and ICAM-2, is a heterodimeric molecule and a member of the integrin family.[8] The regulation of integrins is not yet fully defined but is either $Ca^{2+}$ or $Mg^{2+}$ dependent[9] and their activity may be controlled by various means including phosphorylation[10] and intracelluar second messengers.[11] Therefore lymphocyte interaction with blood–retinal barrier cells will depend upon the regulation of LFA-1 and the expression of counter receptors by RPE and REC.

It has been reported that monoclonal antibodies (mAb) against ICAM-1 can block binding of activated T cells to lymphoid high endothelium,[12] and mAb to ICAM-1, LFA-1 (CD11a) and CD11b/CD18 can inhibit EAU,[13,14] but whether they act by blocking T-lymphocyte activation or inhibiting

Received 1 February 1994; revised 1 April 1994; accepted 9 April 1994.

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lymphocyte migration is unknown. We have previously shown that ICAM-1 expression by human RPE is constitutive and is inducible on retinal microvascular EC[15]and that lymphocyte binding to RPE cells was ICAM-1 dependent, CD2/LFA-3 binding also occurring with REC, but this human assay system was restricted owing to HLA incompatibility. To evaluate the role of integrins and their ligands in lymphocyte activation and extravasation in uveitis we have therefore used the rat model of EAU. Using cultured rat RPE and REC we have investigated the induction and modulation by interferon-$\gamma$ (IFN-$\gamma$) of ICAM-1 and MHC class II molecules and tested the functional relevance of these molecules both in the adhesion of normal and Con A-activated T cells and in retinal antigen-specific lymphocyte proliferation.

## MATERIALS AND METHODS

*Cell culture*

A modification of the method routinely used in this laboratory for the isolation of bovine REC was adapted for rat REC.[16] Briefly, retinae from eight to 10 PVG rats, 2–4 weeks old, were placed in 1 ml of collagenase type IV (0·22 U/ml in GMEM) and homogenized by hand for 1–2 min and a further 1 ml collagenase was added and incubated with interval mixing at 37° for 1 hr 45 min. The disrupted cells were washed and resuspended in Hams F-10 medium (Gibco, Paisley, U.K.) containing 75 $\mu$g/ml endothelial cell growth supplement, 40 $\mu$g/ml heparin, 2 mM glutamine, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, 5 $\mu$g/ml vitamin C, 20% bovine serum and 25% bovine retinal endothelial cell conditioned medium in a 50 $\mu$g/ml collagen type I-coated well. The cultures were maintained at 37° in 5% $CO_2$, and fed every 3–5 days. Primary cultures of capillary REC appeared as small groups, each containing few REC. New cells grew out in circular rows. In the second passage, REC formed regular monolayers with 'cobblestone' morphology (Fig. 1a and b). Indirect immunofluorescence staining with antiserum to human factor VIII revealed the presence of antigen on REC grown on glass coverslips. Staining was localized in cytoplasmic granules that were frequently concentrated in the prenuclear space. REC grown on glass coverslips were also positive by immunofluorescence for uptake of fluorescein-labelled acetylated low-density lipoprotein (AcLDL). RPE cells were isolated from the eyes of 2–4-week-old PVG rats as previously described by Mayerson *et al.*[17] Cytokeratins were demonstrated in the RPE cells (Serotec, Kidlington, Oxford, U.K.). Twenty-four or 96 hr prior to use in the assays REC and RPE cell growth medium on the confluent monolayers were replaced by Ham-F10 and GMEM with 2% normal rat serum respectively. For the induction of adhesion molecules cells were treated with 500 U/ml rat IFN-$\gamma$ (Holland Biotechnology, Leiden, the Netherlands).

*Immunocytochemistry*

Alkaline phosphatase–anti-alkaline phosphatase method (APAAP) was used to detect the presence of phenotypic and activation markers and adhesion molecules on cytospins of T lymphocytes and coverslip cultures of normal and IFN-$\gamma$-stimulated REC and RPE cells. In controls the primary mAb was replaced by irrelevant mAb of similar isotype. Two hundred cells were counted on each slide and the positively stained cells expressed as a percentage.

*Immunization*

Age- and weight-matched PVG rats were immunized with retinal extract (RE, 7 mg/ml) prepared as previously described,[18] emulsified with an equal volume of complete Freund's adjuvant (CFA) containing 1 mg/ml *Mycobacterium tuberculosis* (H37a, Difco Laboratories) and a maximum of 0·1 ml injected into one hind foot pad. One microgram in 500 $\mu$l of the additional adjuvant *Bordetella pertussis* in pyrogen-free PBS (Porton Products Ltd, Maidenhead, U.K.) was also injected i.p. at the same time. Control animals were injected with saline in CFA or saline only.

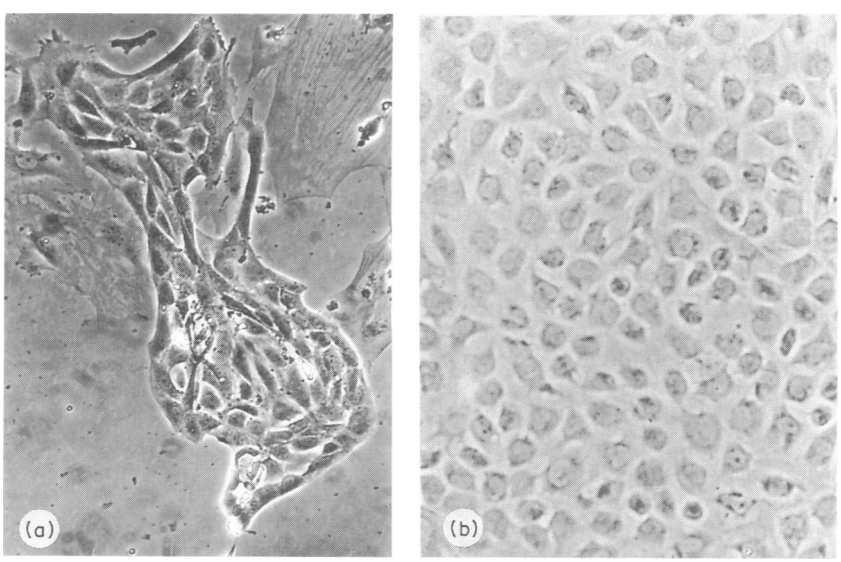


**Figure 1.** (a) Primary cultures of capillary REC appeared as small groups, each containing few REC (magnification ×97·5); (b) REC formed regular monolayers with 'cobblestone' morphology (magnification ×162·5).

*Preparation of T lymphocytes*

T lymphocytes were isolated from the spleens of 8–10-week-old PVG rats. Single-cell suspensions were prepared and lymphocytes isolated by density gradient centrifugation. Macrophages were removed by adherence to tissue grade plastic for 2 hr, and an enriched T-cell population obtained by passage over a nylon wool column.[19] Viability of cells was checked with trypan blue exclusion methods. The cells were immunophenotyped at each stage with both flow cytometry and APAAP immunoenzyme method on cytospins to check for an enriched population of T cells.[20] Cells prepared by this method were 90% OX34 (CD2) positive and 77% OX19 (CD5) positive with residual B cells and dendritic cells available as antigen-presenting cells. For the functional studies, rat T cells were activated with either Con A (2·5 μg/ml), phytohaemagglutinin (PHA; 10 μg/ml) or retinal extract (10%). Flow cytometry analysis of Con A-activated T cells did not show any change in the expression of LFA-1 (91%) or ICAM-1 (22%) molecules in comparison to normal T cells.

*Monoclonal antibodies*

1A29 mAb to rat ICAM-1 (IgG1) and WT.1 to LFA-1α (IgG2a) were purchased from Serotec and dialysed before use to remove sodium azide. OX34 (IgG2a) mAb to rat CD2, OX6 (IgG1) to rat class II and OX60 (IgG1) mAb to an irrelevant antigen were prepared from hybridoma cell lines which were provided by the European Collection of Animal Cell Cultures. The cell lines were grown in culture and the supernatant was collected and filtered before use. The concentration of mAb was measured by radial immunodiffusion test. ED7 and ED8 mAb to rat CD11b (both IgG1, ascitic fluid) were a generous gift from Dr C. Dijkstera, Amsterdam, the Netherlands.

*Aggregation assays*

Functional activity of mAb against rat adhesion molecules was also assessed by virtue of their ability to inhibit Con A- induced lymphocyte aggregation. $1 \times 10^5$ T cells/well in 50 μl of RPMI containing 5% FCS were cultured in flat-bottomed 96-well plates. The cells were exposed to 25 μl (final dilution 2·5 μg/ml) Con A and dilutions of mAb (25 μl) for at least 4 hr. The degree of aggregation inhibition was observed under microscope and scored qualitatively.

*Adhesion assays*

Confluent monolayers of REC and RPE cells grown in 96-well microtitre plates stimulated for 24 and 96 hr with 500 U/ml IFN-γ or cultured in medium containing 2% normal rat serum alone. Preliminary experiments indicated that percentage binding of unactivated T cells to normal RPE and REC varied in a linear fashion and $1 \times 10^5$ T cells were used in the assays. T cells were used either unactivated or in some experiments stimulated with Con A (2·5 μg/ml) for 20 min just prior to chromium labelling. The cells were then washed and suspended at $10^7$ cells/ml and labelled with 200 μCi $Na_2{}^{51}CrO_4$ (Amersham International, Amersham, U.K.) at 37° for 60 min. The labelled lymphocytes were washed three times and resuspended in RPMI medium. The medium on the REC and RPE monolayers was removed, washed and $1 \times 10^5$ $^{51}$Cr-labelled T cells in 50 μl medium were added to each well. In some experiments mAb were added (50 μl). The plates were incubated at 37° for 1 hr. The non-adherent cells were then removed by washing three times with warm medium and the remaining adherent lymphocytes were lysed with 1% triton. The samples from each well and from the original T-cell suspension were counted in a gamma-counter (Packard, Lombard, IL), and the percentage of lymphocytes adhering in each well was calculated using the following formula:

$$\% \text{ adhesion} = \frac{\text{test sample} - \text{counter background}}{\text{total input} - \text{spontaneous release}} \times 100$$

Significance levels were determined using two-tailed Student's *t*-test, a significance level of $P < 0{\cdot}05\%$ was considered significant.

*Proliferation assays*

Spleen lymphocytes from RE-immunized rats were incubated in round-bottomed 96-well plates in triplicate at $2 \times 10^5$ cells/well in 0·1 ml of RPMI containing 5% FCS. 0·1 ml of PHA, Con A or retinal extract in RPMI was added to each well (using optimal stimulatory concentrations) with or without mAb and incubated for 72 hr at 37°. Sixteen hours before harvesting the cells were pulsed with 0·5 μCi [$^3$H]thymidine/well (Amersham) and incubated at 37° in 5% $CO_2$ in air. Using a semi-automatic cell harvester (Skatron, Suffolk, U.K.) cells were harvested onto a filter paper and the incorporation of [$^3$H]thymidine estimated using a beta-counter (Canberra Packard Ltd, Pangbourn, U.K.).

## RESULTS

### Expression of accessory molecules ICAM-1 and MHC class II on REC and RPE cells

The results of immunocytochemical staining of normal and IFN-γ-stimulated RPE and REC using a panel of mAb to accessory molecules are shown in Tables 1 and 2. Normal RPE cells were found to express high levels of ICAM-1 constitutively. Up to 90% of RPE were positive for ICAM-1. Considerably lower levels of ICAM-1 were detected on normal REC (17%) and expression on these cells was weaker than on RPE. Stimulation of both cell types with 5, 50, 100 and 500 U/ml IFN-γ for 24 hr increased the intensity of ICAM-1 expression on over 90% of RPE cells and induced expression of ICAM-1 on 65% of REC. Stimulation of both cell types with the same range of IFN-γ for 96 hr increased the intensity of ICAM-1 expression even further on 95% of RPE cells and induced expression on 80% of REC.

Normal RPE and REC did not express MHC class II. Induction of MHC class II on both RPE and REC was found

**Table 1.** Accessory molecule expression by normal and IFN-γ-stimulated RPE

| | % antigen-positive cells by IFN-γ treatment | | | | |
|---|---|---|---|---|---|
| mAb | 0 | 5 U/ml | 50 U/ml | 100 U/ml | 500 U/ml |
| OX6 (96 hr) | 0 | 0 | 70 | 90 | 90* |
| ICAM-1 (24 hr) | 90 | 90 | 90* | 90* | 90* |
| ICAM-1 (96 hr) | 90 | 90 | 95 | 95 | 95* |

* Increased intensity of expression.

**Table 2.** Accessory molecule expression by normal and IFN-γ-stimulated REC

| mAb | % antigen-positive cells by IFN-γ treatment | | | | |
|---|---|---|---|---|---|
| | 0 | 5 U/ml | 50 U/ml | 100 U/ml | 500 U/ml |
| OX6 (96 hr) | 0 | 0 | 15 | 30 | 35 |
| ICAM-1 (24 hr) | 17 | 65 | 65 | 65 | 65 |
| ICAM-1 (96 hr) | 50 | 65 | 80 | 80 | 80 |

to be dose dependent. Maximal concentration of IFN-γ (500 U/ml) induced MHC class II on 90% of RPE and only 35% of REC after 96 hr incubation with the cytokine, indicating that RPE cells were more responsive to MHC class II induction than REC.

### Functional analysis of novel mAb to rat adhesion/accessory molecules

The active form of LFA-1 can be induced with non-specific mitogens such as Con A which mimics the effects of lymphocyte activation by specific antigen. Functional activity of mAb against rat adhesion molecules and their optimal concentrations were assessed by virtue of their ability to inhibit Con A-induced lymphocyte aggregation. As indicated in Table 3, mAb to ICAM-1, LFA-1 and CD18 effectively blocked lymphocyte cluster formation. OX34 did not block aggregation and no cluster inhibition by OX6 was observed.

### T-cell adhesion to normal and IFN-γ-treated REC and RPE and the effect of mAb

Monoclonal antibody blocking studies were used to define the contribution of LFA-1 and ICAM-1 in binding of unactivated T lymphocytes to RPE and REC. Because CD2/LFA-3 binding is known to occur between T cells and endothelial cells,[21] OX34 (mAb to CD2) was also included in these experiments. As Fig. 2 shows, 40% and 48% of T lymphocytes bound to unstimulated RPE and REC respectively. The adhesion to RPE and REC was not significantly inhibited by 1 : 50 (20 μg/ml IgG) or 1 : 100 (10 μg/ml IgG) dilutions of ascitic fluid mAb to LFA-1 and ICAM-1, suggesting that the adhesion is not ICAM-1/LFA-1 dependent under these conditions. OX34 (70 μg/ml tissue culture supernatant) did not block adhesion. Also when the adhesion assay was carried out at 4°, lymphocyte binding to normal RPE and REC was not inhibited significantly (data not shown).

Although pretreatment of RPE and REC with IFN-γ for 24 and 96 hr enhanced ICAM-1 intensity on RPE and induced the molecule on REC (Tables 1 and 2), these phenotypic changes in adhesion molecule expression on activated REC and RPE did not associate with a significant increase in binding of normal T lymphocytes (data not shown).

**Table 3.** Functional assessment of mAb in inhibition of lymphocyte cluster formation

| mAb | Aggregation score (Con A) |
|---|---|
| Medium | + + + + |
| Anti-ICAM-1 (1 : 100) | + |
| Anti-LFA-1 (1 : 100) | + |
| Anti-CD18 (1 : 3) | + |
| Anti-CD2 (OX34) (1 : 3) | + + + + |
| Anti-MHC II (OX6) (1 : 3) | + + + + |

Aggregation was scored visually where: (0) essentially no cells were in small clusters; (+) < 5% of cells were in small clusters; (+ + + +) > 70% were in small/large clusters.

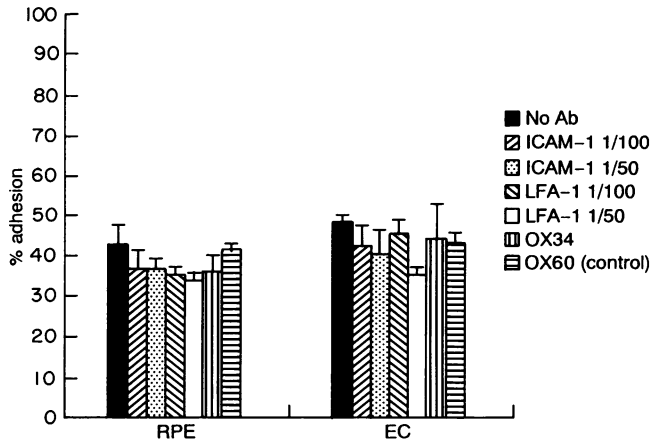


**Figure 2.** Adhesion of normal RPE and REC to normal lymphocytes and the effect of mAb.

### Effect of Con A stimulation of T cells on adhesion to RPE and REC and the effect of mAb

As shown in Fig. 3 activation of lymphocytes with Con A resulted in a marked increase in adhesion to normal RPE (from 42 to 87%) and REC (from 52 to 83%). LFA-1 mAb significantly ($P < 0.0001$) blocked the adhesion of activated lymphocytes to normal RPE (35%) and REC (30%) and ICAM-1 mAb blocked adhesion to both RPE and REC by 20% ($P < 0.001$). OX34 mAb did not significantly inhibit adhesion to RPE and REC.

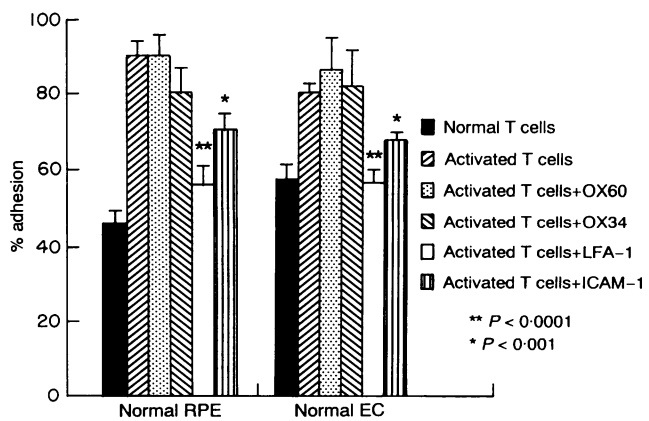


**Figure 3.** Effect of mAb on adhesion of activated lymphocytes to normal RPE and REC.

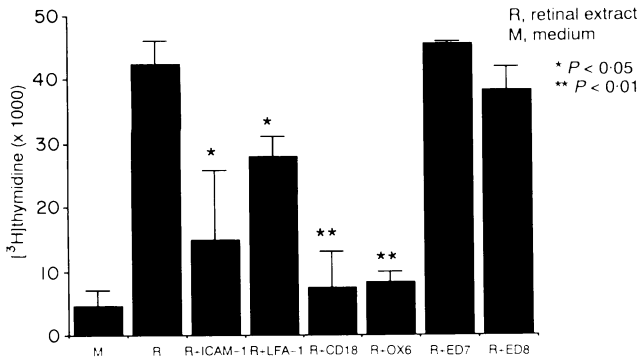


**Figure 4.** Effect of mAb on proliferation of RE-immunized rat spleen T cells. T cells from an RE-immunized rat were cultured alone or with retinal extract as described in the Materials and Methods. Monoclonal antibodies were added at 10 $\mu$g/ml (ED7), 15 $\mu$g/ml (ED8), 20 $\mu$g/ml (LFA-1, ICAM-1 and CD18) and 1 : 3 dilutions of supernatant (OX6). Results are expressed as means ± SD of triplicate samples and significance calculated by Student's *t*-test.

### Effects of mAb against rat adhesion molecules in proliferation of lymphocytes immunized with retinal extract

The role of adhesion/activation molecules in T-cell activation was analysed by the ability of mAb to block proliferation of lymphocytes from retinal antigen-immunized rats. As Fig. 4 demonstrates, RE induced significant proliferation of primed lymphocytes. Inclusion of OX6 (1 : 3 dilutions of supernatant) in the same assay, however, caused inhibition of proliferation demonstrating the MHC class II restriction of the response to retinal extract. The results also showed that mAb to ICAM-1, LFA-1 and CD18 significantly blocked proliferation ($P < 0{\cdot}05$), highlighting the importance of these molecules in regulation of antigen presentation. This inhibition of proliferation was not the result of non-specific antibody effects as proliferation was not inhibited by the mAb ED7 (10 $\mu$g/ml) and ED8 (15 $\mu$g/ml) which recognize different epitopes on the same antigen, CD11b/CD18.

## DISCUSSION

Our study has shown that activation of T-lymphocyte LFA-1 is a critical event governing the adhesion of T cells to RPE and REC as up-regulation of RPE and REC ICAM-1 expression did not increase binding of resting T lymphocytes. However ICAM-1 and CD18 were shown to be required for effective presentation of specific antigen to T lymphocytes.

The levels of adhesion between either normal REC and RPE cells and normal lymphocytes in our experiments were 48% and 40% respectively. This interaction was found to be independent of ICAM-1 and LFA-1 despite constitutive ICAM-1 expression by the RPE cells and probably also to be independent of CD2/LFA-3 as it was not blocked by OX34. It may possibly represent selectin adhesion. Although IFN-$\gamma$ increased ICAM-1 expression on RPE and induced the molecule on REC, no significant alterations in RPE and REC adhesiveness to normal lymphocytes were noted. This may have been the result of secretion of soluble ICAM-1 by the stimulated RPE and REC. ICAM-1 is released by activated human endothelial cells *in vitro* [22] and is reported to inhibit cell adhesion.[23]

The enhanced adhesion of activated lymphocytes (but not resting lymphocytes) to normal and IFN-$\gamma$-treated RPE and REC and its inhibition by LFA-1 mAb highlights the significance of activation in integrin function as a key event in the initiation of T-cell adhesion. The integrin function is cation dependent.[24] Mitogen stimulation of lymphocytes does not increase the expression of LFA-1 on the T-cell surface[11,25] (which is also supported by our data) but Con A can indirectly stimulate protein kinase C and phosphorylation of the $\beta$ subunit altering the configuration of the $\alpha\beta$ heterodimer in the membrane.[26] This may result in the displacement of $Ca^{2+}$ from the $\alpha$ subunit and the acquisition of the $Mg^{2+}$ bound active state.

It has been reported that adhesion of activated T lymphocytes to unstimulated endothelial cells is predominantly ICAM-2 dependent[4] and therefore it may be speculated that activation of lymphocytes enhances the avidity of LFA-1 for ICAM-2, accounting for the increased inhibition of binding obtained with the anti-LFA-1 antibody over anti-ICAM-1 antibody used in these experiments.

We were unable to inhibit the binding of activated lymphocytes to normal or activated RPE or REC with mAb to CD2 (OX34). Similar results have been reported with brain endothelium.[27] However, the interaction between CD2 and LFA-3 is suggested to have a relatively low affinity[4] and its interaction with other cells appears to be influenced by the surface charge/glycocalyx density. It is possible that the binding of CD2 antibody to the T cells resulted in activation of the T cells which would result in increased LFA-1-dependent binding. As CD2/LFA-3 binding is also T-lymphocyte activation dependent a proportion of the observed increased binding (following T-cell activation) might also be accounted for by this interaction, but it is likely that other adhesion pathways are in operation such as VCAM-1/VLA-4 (vascular cell adhesion module-1/very late antigen-4) which remain to be elucidated.

The results of proliferation assays indicate that the presence of mAb against ICAM-1 and LFA-1 adhesion molecules probably inhibited co-stimulatory signals between APC and T cells. In addition to the failure of antigen presentation, the absence of APC/T cell interaction may affect the cells in two other ways. Firstly inhibition of IL-1 induction as a co-stimulator may occur, and secondly inhibition of secretion of an IL-1-inducing lymphokine such as tumour necrosis factor by T cells may also occur.[28] The results also suggest that ED7 and ED8 epitopes of the C3bi complement receptor (CD11b/CD18) do not play a role in antigen presentation, although mAb specific to the CD18 common $\beta$ chain was highly effective in blocking T-cell proliferation.

Monoclonal antibodies to integrins can inhibit EAU and endotoxin-induced uveitis (EIU) in the rat,[13,14] but the mechanisms involved are not known. Our results suggest that modulation of disease may be achieved at the level of antigen presentation as well as blockade of adhesion. It has been shown that co-stimulatory signals are required for T-cell receptor (TCR)-mediated activation of T lymphocytes and lack of co-stimulation can result in T-cell inactivation.[29] It has been suggested by other investigators that LFA-1 may be involved in this co-stimulatory signal on binding to ICAM-1.[30] Our study has shown that activation of T-lymphocyte LFA-1 (CD11a) is a critical event governing the adhesion of activated T cells to RPE and REC, and that CD18 and ICAM-1 in addition to

CD11a are required for effective antigen presentation. These results support the hypothesis that antigen-independent adhesion precedes TCR engagement (Fig. 2), that additional adhesion molecule interactions which increase in avidity on T-cell activation occur (Fig. 3), and that TCR interactions with the MHC–peptide complex require subsequent co-stimulation via integrins (Fig. 4). As no long-term culture assays are available for human systems, our rat model provides an opportunity to study the interaction of T-cells with cells of the blood–retina barrier and identify the mechanisms which result in T-cell activation and extravasation, invaluable information in the development of new therapeutic strategies.

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